Journal of Chromatography, 225 (1981) 231—235
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 905

Note

Efficient extraction and reversed-phase high-performance liquid chromatography—ultraviolet quantitation of acetazolamide in serum

DONALD M. CHAMBERS*, MARTHA H. WHITE and HARRY B. KOSTENBAUDER

Division of Pharmaceutics and Pharmaceutical Analysis, College of Pharmacy, University of Kentucky, Lexington, KY 40506 (U.S.A.)

(Received February 18th, 1981)

Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) is a carbonic anhydrase inhibitor which reduces the rate of aqueous humor formation and correspondingly decreases the intraocular pressure in patients with glaucoma. Recent methods for the quantitation of acetazolamide in biological fluids have included two high-performance liquid chromatography (HPLC) procedures [1, 2], an enzymatic method [3], and an electron-capture gas chromatography procedure [4]. The HPLC methods require extensive and time consuming extraction and evaporation steps [1, 2]. The carbonic anhydrase inhibition assay is plagued with insufficient precision [2, 3]. The method of Wallace et al. [4] is currently considered the method of choice, but Silber [5] has indicated that it would be desirable for a reversed-phase HPLC method to be developed that would only require a simple sample preparation procedure. The latter approach has been chosen. The method developed in our laboratories avoids time consuming evaporation steps, possesses excellent precision, adequate sensitivity and is well-suited for automation.

EXPERIMENTAL

Materials

Chlorothiazide and hydrochlorothiazide were supplied by Merck, Sharp and Dohme (West Point, PA, U.S.A.) and acetazolamide was obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.). Acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) was UV-HPLC grade, citric acid monohydrate (J.T. Baker, Phillipsburg, NJ, U.S.A.) was a Baker analyzed reagent, water was house distilled, and all other chemicals were of reagent grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Serum was obtained from drug-free healthy

0378-4347/81/0000—0000/$02.50

male volunteers. SurfaSil was obtained from Pierce, Rockford, IL, U.S.A. for the purpose of siliconizing the surface of glassware.

Apparatus

A Waters Model 6000A pump and a 710B WISP autosampler were coupled to an Ultrasphere-ODS (5 μm, 4.6 × 250 mm) reversed-phase column (Altex Scientific, Berkeley, CA, U.S.A.). The analytical column was protected by use of a guard column (40 × 4.6 mm I.D.) packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). Absorbance of the eluate was monitored with a Waters 440 UV detector (254 nm). The absorbance output (1 a.u./V) of the detector was connected to both channels of a Houston Superscribe recorder (Houston Instrument, Austin, TX, U.S.A.). Full range recorder spans of 50, 200 and 500 mV were used to provide on-scale peaks.

Mobile phase

A mixture of acetonitrile—0.05 *M* acetate buffer, pH 4.5 (10:90, v/v) was filtered through a 0.45-μm nylon-66 membrane filter (Rainin Instrument, Woburn, MA, U.S.A.) and deaerated under vacuum. The mobile phase was pumped at a rate of 1 ml/min and developed an operating pressure of 2200 p.s.i. (ca. 152 bars).

Stock solutions

Aqueous acetazolamide solutions of 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 15.0 and 20.0 μg per 0.1 ml were stored in amber bottles at 4°C. The internal standard solution was 5 mg of chlorothiazide in a total volume of 2000 ml of ethyl acetate.

Procedures

Culture and tapered centrifuge tubes were treated with a 10% solution of SurfaSil in acetone. Serum (1 ml) was then placed in a 20-ml culture tube. Water (0.1 ml), 1.0 ml of citrate buffer (0.05 *M*, pH 4.6), about 700 mg of sodium chloride, and 10.0 ml of the internal standard solution were added to the sample. The tube was closed with a PTFE-faced screw-cap, vortexed for 1 min and centrifuged for 5 min. Inversion of the tube prior to placing it on the vortex apparatus was helpful in avoiding the formation of an emulsion. If an emulsion formed, it was possible to sonicate the contents of the tube in order to obtain a clear organic phase. The ethyl acetate layer was then transferred to a 13-ml tapered centrifuge tube. To the organic phase was added 0.4 ml of phosphate buffer (0.1 *M*, pH 11.9). The tube was capped, vortexed for 1 min, and centrifuged for 5 min. A 0.2-ml portion of the aqueous phase was transferred to a Waters low-volume insert for automated sample processing and injection of 35 μl.

Standards from 0.05—20 μg/ml were prepared by spiking 1.0 ml of blank serum with 0.1 ml of the appropriate acetazolamide stock solution. The standards were extracted in the same manner as the samples but without the addition of 0.1 ml water. Peak height ratios of acetazolamide to chlorothiazide were plotted versus acetazolamide concentration in μg/ml, and the resulting calibration curve was used to calculate the serum concentrations of the unknown samples.

Absolute recovery of 0.1 and 20.0 μg/ml spiked serum standards (n = 6) were determined from a calibration curve of acetazolamide peak height versus the amount on-column. Two 5.0 μg/ml spiked serum standards were processed without the addition of salt, and the absolute recovery evaluated as above.

An 8 μg/ml control standard was processed daily (n = 12) in order to evaluate the day-to-day reproducibility. Precision was assessed by processing spiked samples (n = 6) of 0.1, .1, 5 and 20 μg/ml and computing the peak height ratios.

Spiked serum samples of 1 and 20 μg/ml (n = 3) were processed and immediately injected. Injections were also made at 4, 8, 12 and 16 h after the initial injection. Peak height ratios were determined in order to validate the method of sample storage.

RESULTS AND DISCUSSION

Chromatograms of blank and spiked human serum are shown in Fig. 1.

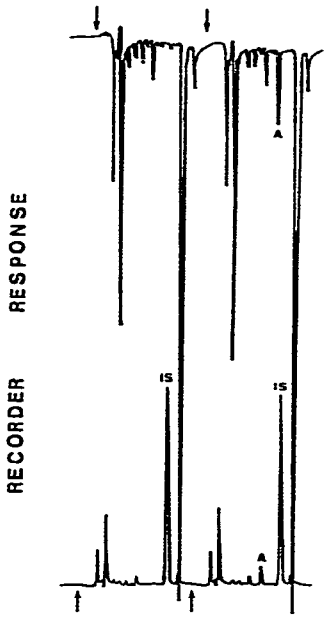

Fig. 1. Simultaneous recording of two full-scale absorbance ranges of 0.05 (upper) and 0.2 (lower) of blank serum and spiked serum (0.5 μg/ml) samples. Arrows mark injection; peaks: A = acetazolamide; IS = internal standard, chlorothiazide.

The retention volumes of acetazolamide and chlorothiazide are approximately 10 and 12 ml respectively and no interfering peaks are present. Hydrochlorothiazide was found to have a retention volume of about 14 ml, and therefore would not interfere with the chromatography.

Calibration curves were linear over the concentration range of 0.05—20 μg/ml and the intercept was essentially zero. A mean correlation coefficient of 0.999 ± 0.0003 was observed for the 12 calibration curves. The practical

limit for the detection of acetazolamide is 0.05 μg/ml.

Absolute recoveries for spiked serum samples (n = 6) of 0.1 and 20 μg/ml were 95.9 ± 1.82% and 97.6 ± 3.40% respectively. Processing of two 5 μg/ml samples without the addition of salt resulted in only a 65.0 ± 0.44% recovery. Previous HPLC assays for acetazolamide [1, 2] have used 10 ml of ethyl acetate as an extraction solvent, but these methods required that the process be repeated to provide almost complete recovery. The addition of sodium chloride to the sample provides 95% recovery of acetazolamide with one 10-ml ethyl acetate extraction.

The within-day precision was evaluated at concentrations of 0.1, 1.0, 5.0 and 20.0 μg/ml (n = 6) and the coefficients of variation (C.V.) were found to be 2.54, 1.12, 1.26 and 1.33%, respectively. Excellent reproducibility between days is also observed with a C.V. of 2.50% (n = 12) for an 8 μg/ml control standard.

During recent years there has been an increase in the usage of automated sample injectors for HPLC. This has allowed a greater number of samples to be processed daily, but also results in an increased period of time between extraction and injection of the sample. Therefore, stability of the sample while stored in the automatic injector should be evaluated. Furthermore, the pH 11.9 aqueous buffer that was used for back extraction and subsequent storage of the sample was of additional concern with regard to stability. It was observed that the pH of the buffer decreased to a pH of less than 9 after back extraction. Therefore, the problem of alkaline degradation of acetazolamide and chlorothiazide was diminished and resulted in essentially constant peak height ratios for up to 16 h (Table I). This period of time would allow the analysis of 64 samples. Beginning analysis of some samples in the middle of the day would provide time for up to 80 samples per day.

TABLE I

EVALUATION OF SAMPLE STORAGE FOR UP TO 16 h DURING AUTOMATED INJECTION

Concentration (μg/ml)	Peak height ratio (mean, n = 5)	C.V. (%)
1	0.1655	1.56
1	0.1682	1.27
1	0.1675	0.98
20	3.337	1.40
20	3.466	2.32
20	3.296	1.43

CONCLUSIONS

The described method is simple, rapid, sensitive and possesses excellent precision. The addition of sodium chloride to the sample obviates a second ethyl acetate extraction, and the back extraction step makes it unnecessary to evaporate the samples to dryness and reconstitute.

This assay is an improvement over previous HPLC acetazolamide assays and is a viable alternative to the assay of Wallace et al. [4] for serum levels of acetazolamide. It has been successfully applied to the analysis of over 500 samples from human volunteers who were administered various acetazolamide dosage forms.

ACKNOWLEDGEMENTS

Donald Chambers is The 1979 and 1980 American Foundation for Pharmaceutical Education Analytical Chemistry Fellow.

This study was supported by a grant from Lederle Laboratories.

REFERENCES

1 W.F. Bayne, G. Rogers and N. Crisologo, J. Pharm. Sci., 61 (1975) 402.
2 R.D. Hossie, N. Mousseau, S. Sved and R. Brien, J. Pharm. Sci., 69 (1980) 348.
3 G.J. Yakatan, C.A. Martin and R.V. Smith, Anal. Chim. Acta, 84 (1976) 173.
4 S.M. Wallace, V.P. Shah and S. Riegelman, J. Pharm. Sci., 66 (1977) 527.
5 B. Silber, in W. Sadee and G.C.M. Beelen (Editors), Drug Level Monitoring: Analytical Techniques, Metabolism and Pharmacokinetics, Wiley, New York, 1980, p. 117.